Romanian Biotechnological Letters *Vol. 21, No. 4, 2016*

ORIGINAL PAPER

Antagonistic Marine Bacteria Associated with *Spongionella gracilis*

Received for publication, November 30, 2014
Accepted, March 1, 2015

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Abstract

Marine sponges are the most biodiverse and biologically productive of all marine ecosystems. Sponges harbor diverse and abundant prokaryotic communities. However, little is known about the diversity of sponge- associated microorganisms. We used molecular techniques to identify and compare the culturable bacterial assemblages associated with Spongionella gracilis collected from Mediterranean Sea. The bacterial communities within sponges were found to be mostly representatives of Firmicutes and few Gammaproteobacteria. Antimicrobial activity of the microbial isolates were tested against four pathogenic bacteria, and two fungi. A relatively high proportion of microbial isolates displayed distinct antibacterial activities and few anticandidal activities, suggesting that sponge associated microorganisms may aid their host in protection against marine pathogens. The genus Bacillus had the highest proportion of antimicrobial activity which supported the hypothesis that Bacillus spp. might play a protective role in the sponge's hosts.

Keywords: *Spongionella gracilis, 16SrRNA, Phylogenetic diversity, Antimicrobial activity,*

1. Introduction

It was shown recently that some bioactive compounds isolated from invertebrates originated from symbiotic microorganisms (1).It has been reported that marine invertebrates harbor a higher population of bacteria able to produce novel bioactive compounds (2). An increase of bacterial resistance to existing antibiotics has led to the search for new drugs, especially antibiotics. Marine microorganisms are currently of considerable interest as a new and promising source of biologically active compounds. They produce a variety of metabolites, some of which can be used for drug development (3).Sponges (Phylum Porifera) are very fertile host animals for diverse symbiotic microorganisms. Sponges are simple multicellular invertebrates attached to solid substrates in benthic habitats. All sponges are filter feeders; numerous tiny pores on the surface allow water to enter and circulate through a series of canals where microorganisms and organic particles are filtered out and eaten. Since sponges are efficient filter feeders, any microorganism that can resist the sponge digestive process and immune response can successfully inhabit sponges (4).Sponges are described as microbial fermenters (5) that harbor diverse and complex assemblages of microorganisms including heterotrophic bacteria, cyanobacteria, facultative anaerobes, unicellular algae, and Archaea (6).Sponge-associated microbes can constitute up to 60% of the sponge biomass (7).As proposed by Taylor *et al*., (2007a), the term "symbiont" is used to describe sponge-associated bacteria. The broadest definition of bacteria, fungi and some of these microbes are probably host-specific. Microbes can compose up to 50% of the sponge tissue volume (8).Numerous studies have reported the antimicrobial activity of a variety of 'extracts' from

various organisms, such as sponges (9), soft corals(10, 11)and scleractinian corals. Sponges are excellent sources for natural products acting as bioactive compounds. Their bioactivity includes enzyme inhibitors, cell division-inhibitors, antiviral, antifungal, antimicrobial, anti-inflammatory, antitumor cytotoxic or cardiovascular properties (12).This work aims to investigate the phylogenetic diversity and antimicrobial activity of culturable prokaryotic communities associated with *Spongionella gracilis.*

2. Materials and Methods

Invertebrate

Sponges were collected from the Red sea (November 2009) and kindly identified as *Spongionella gracilis* (Figure 1)by Dr. **Tarek A. Temraz,** Marine Science Department, Suez Canal University, Ismailia, Egypt. They were collected in sterile plastic bags without seawater, kept at 4ºC and transferred immediately to the laboratory for processing.

Figure.1*Photograph of the Mediterranean Sea sponge Spongionella gracilis*

Indicator microbes

Bacterial pathogens used for the assay of antimicrobial activity were clinical strains of *Staphylococcus aureus, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*, obtained from Medical Research Institute at Alexandria University, and the fish pathogen *Vibrio fluvialis* obtained from National Institute of Oceanography and Fisheries, Alexandria. They were all cultured in Luria Bertani (LB) broth at 37º C. The fungi *Penicillium* sp. and *Aspergillus niger* were provided from the Microbiology Lab, Faculty of Science, Alexandria University. They were cultured in Potato dextrose (PD) broth at 30º C. *Candida albicans* was cultured in Yeast malt extract (YM) broth at 30º C for 48 h. All cultures on the corresponding agar medium supplemented with 15% glycerol (w/v) and stored frozen at 4ºC.

Phylogenetic analysis of culturable bacteria

Six bacterial isolates were subjected to 16SrDNA sequence analysis. The bacterial isolates were grown overnight in 2ml Nutrient Broth. Genomic DNA was extracted with the DNA extraction kit (Fermentas**)** using the manufacturer procedure and analyzed by electrophoresis in 1% agarose gel. The PCR reaction mixture contained 5µl of template DNA, 2µl of bacterial- specific primers Forward 5'CGC GGC CTA TCA CTTGT TG 3', and Reverse 5'CCG TAC TCC CCA GGC GGG G 3', 2µl of dNTP (25µl), 4µl of $MgCl_2$ (25 mM), 5µl of PCR buffer (10x) and 1µl Taq polymerase, distilled water was added to 50µl final volume. The cycling programmer was 95ºC for 5min, 35 cycles at 95º C for 30 sec, 50ºC for 30 sec, and 72º C for 2 min, at the end, the reaction was incubated at 72 ºC for 10 min.

The extracted DNA and PCR products were visualized by UV trans- illuminator after staining the gel with ethidium bromide (10mg/ml) for 20 min.The PCR products were purified using the gene gel purification kit (Fermentas, lithuania) and sequenced by GCAT Company (USA). For each isolate, there were two replicas of PCR product. One was sequenced with the forward primer and the other with the reverse primer .Sequence data were analyzed by comparison to 16S rDNA genes in the Genbank database. The nearest relatives of each organism were obtained by BLAST searches (13).To build the phylogenetic tree, sequences were aligned using the multiple sequence alignment program from MEGA 4 (14) using the ClustalW method. A neighbor joining tree (15) was constructed. The statistical significance of tree branches was evaluated by bootstrap analysis.

Antimicrobial assay

The assay was performed as previously described (16) with some modifications. Each strain was grown on medium of isolation and incubated at 30° C until visible growth. A colony of each strain was then spotted onto the surface of agar plates of corresponding media seeded with indicator strains and incubated at 30°C for 24- 48h. Inhibition zones were scored as antibacterial, antifungal or anticandidal and measured in mm.

3. Results and Discussion

3.1. Phylogenetic diversity of sponges

Marine sponges have been shown by 16S rDNA community analysis to contain remarkably diverse microbial communities that include many novel bacteria that have been found only within sponges (17, 18, 19).The 16S rDNA gene sequences of six bacterial isolates recovered from *Spongionella gracilis* was phylogenetically analyzed. The microbial communities were found to be restricted to two bacterial groups. The bacterial assemblage of the sponge had representatives within the *Gammaproteobacteria* (two strains) and *Firmicutes* (four strains). Table1 shows the accession numbers of isolates and similarity percentages to nearest neighbors. The phylogenetic tree is illustrated in Figure 2. The data obtained in this study showed that the percentage of *Firmicutes* are higher than *Gammaproteobacteria*. This data is dissimilar to the findings of Chelossi *et al*. (22) who implied that 58% of the aerobic heterotrophic bacterial strains isolated from the sponge, *Petrosiaficiformis* were identified as Gram negative. *Gammaproteobacteria* phylum is relatively common and abundant in sea water (20, 21).The finding that the percentage of Gram positive strains was found to be lower (20-27%) than the Gram negative strains (73-80%) (1) agrees with those found by Fenical and Jensen (23) who reported that the bacteria present in seawater were mainly negative rods. Another study revealed that the bacterial strains isolated from various regimens of the marine environment showed that 82.28 % were Gram negative (24). Two strains HS3 and HS4 were assigned to genus *Provedencia*, Phyllum *Gammaproteobacteria*. The first showed 100% similarity to *Provedencia. rustigianii,* whereas the second was 99% similar to *Providencia alcalifaciens* (Table 1, Figure.2). Bacteria of the genus *Providencia* are rods, gram-negative opportunistic pathogens that have been isolated from a wide variety of environments and organisms ranging from humans to insects to sea turtles and shark mouths. Members of the genus have repeatedly been found in association with humans, insects and many other vertebrate and invertebrate animals in both pathogenic and non-pathogenic contexts (25).

A strain identified as *Provedencia* sp. was isolated from the sponge *Jaspis* sp. (26). As regards to aquaculture, *Providencia rettgeri* has been associated with mass mortalities of carp

(*Hypophthalmichthysmolitrix*) in Israel. The organism was isolated from internal organs and skin ulcers in fish affected of severe sepsis. Subsequently, in its presence in the feaces of sea turtles (*Caretta caretta*) (27) was described. *Providencia stuartii* has been described like resistant to all β-lactams including carbapenems .They encoded a novel metallo-β-lactamase which was inhibited by EDTA and hydrolyzed penicillins, cephalosporins, and carbapenems (28).

The identified Gram positive strains belonged to Phyllum *Firmicutes*, Genus *Bacillus. B.* sp HS1, *Bacillus subtilis* HS2, and *B. amyloliquefaciens* HS6 were 100% similar to *B.* sp TSH22w, *Bacillus subtilis* strain F121112 and; B. *amyloliquefaciens* wh2*,* respectively whereas *Bacillus subtilis* HC5was 99% similar to *B.subtilis* SSCS9.

Table 1. 16S rDNA of bacterial strains isolated from sponge, their accession number and their similarity percentage to the closest neighbor.

Identification	Accession Number	Homology strain	Homology percentage	Phylum
Bacillus sp HS1	JQ929073	*Bacillus* sp. TSH22w gene for 16S ribosomal RNA, partial sequence	100%	Firmicutes
*Bacillus subtilis*HS2	JQ929074	*Bacillus subtilis* strain F121112 16S ribosomal RNA gene, partial sequence	100%	Firmicutes
Providencia rustigianii HS3	JQ929075	*Providencia rustigianii* 16S rRNA gene, type strain DSM 4541	100%	GammaProteobacteria
Providencia alcalifaciens HS4	JQ929076	*Providencia alcalifaciens* strain G79 16S ribosomal RNA gene, partial sequence	99%	GammaProteobacteria
*Bacillus subtilis*HS5	JQ929077	*Bacillus subtilis* gene for 16S rRNA, partial sequence, strain: SSCS9	99%	Firmicutes
*Bacillus amyloliquefaciens*HS6	JQ929078	*Bacillus amyloliquefaciens* strain wh2 16S ribosomal RNA gene, partial sequence	100%	Firmicutes

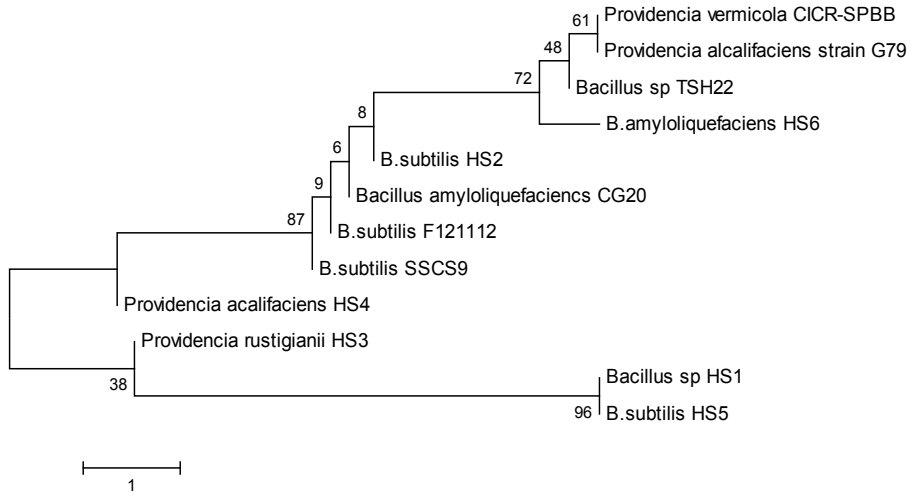


Figure. 2 Neighbor-joining phylogenetic tree bacterial isolates isolated from *Spongionella gracilis* . The numbers at nodes are percentages indicating the levels of bootstrap support, based on a neighbour-joining analysis. The scale bar represents 0.1 substitutions per nucleotide

3.2. Antimicrobial activity of the sponge *Spongionella gracilis* associated bacteria

In the marine environment, members of the genus *Bacillus*, are known for the production of metabolites with antimicrobial, antifungal or generally cytotoxic properties. They were regularly isolated from invertebrates and thus display a high potential in the search for new antimicrobial substances (29, 30, 31). Therefore, the antimicrobial activity of the six strains isolated in this study from the *Spongionella gracilis* was evaluated. Only one strain (*Bacillus subtilis* HS2) exhibited antimicrobial activity against three pathogens namely *S.aureus*, *K.pneumoniae* and *C.albicans*. *Bacillus* sp HS1 was active only against *S.aureus* and *C. albicans*, whereas *B.amyloliquefaciens* HS6 showed activity against *S.aureus* only (Table 2, Figure 3, Figure 4). The other two sponge-associated bacterial strains *Providencia alcalifaciens* HS4 and *Providencia rustigianii* HS3 found in our study were of limited activity. Inhibitory activity was observed against important pathogenic species such as *S. aureus and K. pneumoniae*. The genus *Providencia* can be found commonly in soil, water, and sewage (32). In a recent study (26), a protease inhibitor of *Providencia* was isolated from sponge. The high frequency of activity against Gram-positive bacteria was expected, since Gram-negative bacteria are generally less susceptible to antimicrobials than Gram-positive bacteria because of the presence of an outer membrane and lipopolysaccharide (LPS) which together act as an efficient barrier against hydrophobic and lipophilic molecules (33). To our knowledge, no reports are available on the antimicrobial production by members of genus *Providencia* isolated from Mediterranean or Red sea in Egypt.

Table 2. Antimicrobial activity of bacterial isolates from *Spongionella gracilis* against different pathogens represented by the size of inhibition zone (mm).

Strains	*S.aureus*	*V.fluvialis*	*K.pneumoniae*	*P.aeruginosa*	*C.albicans*	*Penicillium sp*	*A.niger*
***Bacillus* sp HS1**	12	-	-	-	13	-	-
***Bacillus subtilis* HS2**	20	-	15	-	18	-	-
***Providencia rustigianii* HS3**	30	-	13	-	-	-	-
***Providencia alcalifacien*s HS4**	7	-	-	-	-	-	-
***Bacillus subtilis* HS5**	12	-	18	-	-	-	-
***Bacillus amyloliquefacie ns*HS6**	9	-	-	-	-	-	-

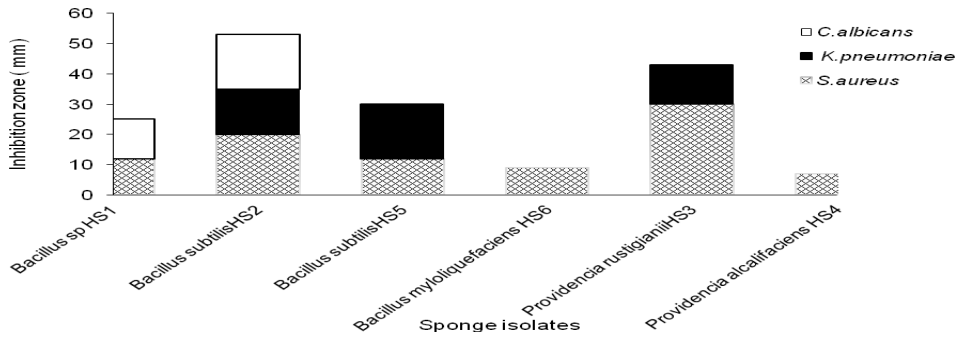
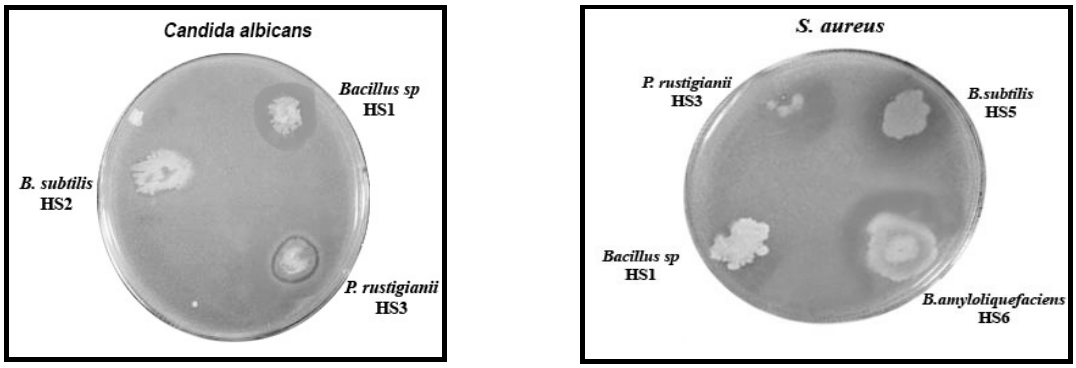


Figure. 3. Bioassay of bacterial strains isolated from the *Spongionella gracilis* against indicator strains measured as inhibition zone (mm).



(A) **(B)**

Figure. 4. Agar diffusion plates showing anticandidal (A) and antibacterial (B) of some selected bacteria from *Spongionella gracilis*.

Conclusion

Based on results obtained in this work, it can be concluded that member sof Firmicutes and Gamma Proteobacteriaare associated within *Spongionella gracilis*. Isolated strains possess a broad spectrum of antimicrobial activity and could be of great potential in producing novel antimicrobial agents.

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